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Novel DNA-Binding Proteins in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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As an approach towards elucidation of the biochemical regulation of the progression of heterocyst differentiation in *Anabaena* sp. strain PCC 7120, we have identified proteins that bind to a 150-bp sequence upstream from *hepC*, a gene that plays a role in the synthesis of heterocyst envelope polysaccharide. Such proteins were purified in four steps from extracts of vegetative cells of *Anabaena* sp. Two of these proteins (Abp1 and Abp2) are encoded by neighboring genes in the *Anabaena* sp. chromosome. The genes that encode the third (Abp3) and fourth (Abp4) proteins are situated at two other loci in that chromosome. Insertional mutagenesis of *abp2* and *abp3* blocked expression of *hepC* and *hepA* and prevented heterocyst maturation and aerobic fixation of N_2.

Cyanobacteria are microorganisms that require only light as a source of energy and water as a source of reductant. These bacteria have been used to study cell division and differentiation, photosynthesis, nitrogen fixation, hydrogen metabolism, and responses to environmental changes. *Anabaena* sp. strain PCC 7120, a filamentous cyanobacterium, is simultaneously capable of oxygenic photosynthesis and nitrogen fixation. Nitrogen fixation is closely associated with cellular differentiation and intercellular interactions. In the presence of ammonium or nitrate, filaments of *Anabaena* sp. are comprised solely of photosynthetic vegetative cells. When deprived of a source of fixed nitrogen, vegetative cells differentiate at semiregular intervals along the filaments into heterocysts, cells specialized for the fixation of N_2. The nitrogen-fixing activity of heterocysts depends upon products of photosynthesis that they receive from neighboring vegetative cells, to which, in return, heterocysts supply products of nitrogen fixation. The differentiation of heterocysts involves extensive biochemical and structural changes that are regulated by a cascade-like sequence of transcriptional activations whose coordination is little understood (9, 48).

Regulatory DNA-binding proteins that are known to affect heterocyst formation in *Anabaena* sp. include a histone-like protein, HU (27); transcription factor cyclic AMP receptor protein-related proteins NtcA (16, 45) and DevH (21); and PatB, a ferredoxin-like protein with a helix-turn-helix motif (33). However, RNA polymerase sigma factors SigA, which is expressed in both the presence and absence of combined nitrogen, and SigB and SigC, which are expressed transiently under nitrogen-limiting conditions, and five additional sigma factors (SigD, SigE, SigF, SigG, and SigH) are not involved specifically in the regulation of heterocyst differentiation (6, 7; I. Khudyakov, X. Wu, and J. W. Golden, Abstr. 10th Int. Symp. Phototroph. Prokar., p. 193, 2000.). HU is degraded during heterocyst differentiation, and a new, similar but different protein is synthesized in the heterocyst (37). Mutation of *hanA*, which encodes HU, results in a highly pleiotropic phenotype that includes slow growth, altered pigmentation, resistance to phage A-4(L), cellular fragility, and the inability of heterocysts to differentiate (27). NtcA binds upstream from *xisA*, which encodes a site-specific recombinase, but also upstream from *glnA*, *rbcL*, *nifH*, and *hetC* (10, 22, 36) and is required for heterocyst differentiation in *Anabaena* sp. (16, 36, 46). Despite this accumulation of known factors, the regulatory mechanisms that underlie the progression of differentiation in cyanobacteria remain largely unknown (48).

Earlier, *hepB*, *hepK*, and neighboring genes *hepC* and *hepA* were shown to be required for the normal formation of a principal structural element of heterocysts, the outer, polysaccharide layer of their envelope (48, 50). A DNA sequence upstream from *hepC* greatly influences induction of *hepA* upon nitrogen stepdown (50). We have identified four proteins that bind to a 150-bp sequence, X12, upstream of *hepC* and have shown that two of these proteins play an important role in the differentiation of heterocysts and in their ability to fix N_2 aerobically.

MATERIALS AND METHODS

Bacterial strains and culture conditions, plasmids, and manipulation of DNA. *Anabaena* sp. strain PCC 7120 and its derivatives (Table 1) were grown at 30°C in light (ca. 3,500 ergs cm^{-2} s^{-1}) on a rotary shaker in AA/8 medium (24) either supplemented with 5 mM nitrate in 125-ml Erlenmeyer flasks or not supplemented or, for extraction of proteins, in 3-liter batches in 6-liter flasks that were bubbled continuously with sterile air. For preparation of RNA, cyanobacterial cells were grown in 4 liters of AA/8 medium with 5 mM nitrate for 5 days. Cells were harvested from 500 ml of the suspension. The remaining cells were sedimented (1,000 × *g* to 1,800 × *g*) at room temperature (ca. 23°C); washed thrice with fresh AA/8 medium; resuspended in 3.5 liters of fresh, nitrate-free AA/8 medium under the same conditions of light and temperature as before; and harvested in 500-ml portions after 0, 3, 6, 10, 24, 30, and 48 h of nitrogen deprivation. Derivative strains were grown in the presence of appropriate antibiotics. Plasmids (Table 1) were introduced by conjugation (14). Total DNA was extracted from cells of *Anabaena* sp. as described by Koksharova et al. (29). *Escherichia coli* strain DH10B (20) was grown in Luria-Bertani medium with antibiotics appropriate for resident plasmids. Routine procedures were used for manipulation of DNA, plasmid transformation of *E. coli*, and selection and testing of transformants (41). Enzymes were purchased principally from New England BioLabs (Beverly, Mass.) and used as recommended by the manufacturer.

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TABLE 1. Cyanobacterial strains and plasmids

Anabaena strain or plasmid	Derivation and/or relevant characteristics[a]	Source or reference
Strains		
PCC 7120	Wild type	R. Haselkorn
ABP1	Nm^r, *abp1* homologous recombinant of pRL2469 with chromosomal DNA of *Anabaena* sp. strain PCC 7120	This study
ABP2	Nm^r, *abp2* homologous recombinant of pRL2482 with chromosomal DNA of *Anabaena* sp. strain PCC 7120	This study
ABP3	Nm^r, *abp3* homologous recombinant of pRL2486 with chromosomal DNA of *Anabaena* sp. strain PCC 7120	This study
ABP4	Nm^r, *abp4* homologous recombinant of pRL2470 with chromosomal DNA of *Anabaena* sp. strain PCC 7120	This study
CPB8226	Em^r Nm^r Sm^r Sp^r, *abp2* homologous recombinant of pRL2482 with chromosomal DNA of strain SR1069a	This study
CPB8563	Em^r Nm^r Sm^r Sp^r, *abp3* homologous recombinant of pRL2486 with chromosomal DNA of strain SR1069a	This study
SR1069a	Em^r Sm^r Sp^r, single recombinant of wild-type *Anabaena* sp. strain PCC 7120 with pRL 1069a	3
Plasmids		
pBS-SK(+)	Ap^r, cloning vector	Stratagene, La Jolla, Calif.
pET-21b	Ap^r, expression vector	Novagen, Madison, Wis.
pRL498	Km^r, positive selection cloning vector	12
pRL623	Cm^r, helper plasmid bearing methylase genes *avaiM, eco47iiM*, and *ecot22iM*	14
pRL1069a	Cm^r Em^r Sm^r Sp^r, *hepA*::*luxAB*	3
pRL2469	Km^r, pRL498 cut with *Sma*I and ligated to the blunted ends of a PCR copy of truncated *abp1*	This study
pRL2470	Km^r, pRL498 cut with *Sma*I and ligated to the blunted ends of a PCR copy of truncated *abp4*	This study
pRL2475	Ap^r, pBS-SK(+) cut with *Pst*I and ligated to a *Pst*I-digested fragment of a PCR copy of truncated *abp3*	This study
pRL2482	Km^r, pRL498 cut with *Sma*I and ligated to the blunted ends of a PCR copy of truncated *abp2*	This study
pRL2486	Km^r, pRL498 cut with *Eco*RI and *Xba*I and ligated to the smaller *Eco*RI-*Xba*I fragment of pRL2475	This study
pRL2489	Ap^r, *abp1* PCR-amplified and cloned between the *Nde*I and *Bam*HI sites of pET-21b	This study
pRL2490	Ap^r, *abp2* PCR-amplified and cloned between the *Nde*I and *Bam*HI sites of pET-21b	This study
pRL2491	Ap^r, *abp3* PCR-amplified and cloned between the *Nde*I and *Bam*HI sites of pET-21b	This study
pRL2492	Ap^r, *abp4* PCR-amplified and cloned between the *Nde*I and *Bam*HI sites of pET-21b	This study

[a] Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Nm, neomycin; r, resistant; Sm, streptomycin; Sp, spectinomycin.

Purification of proteins from *Anabaena* sp. Three-liter cultures of *Anabaena* sp. in AA/8 medium with nitrate were harvested by centrifugation at 4°C for 20 min at 5,000 × *g*. Cells were washed with cold 25 mM HEPES-KOH (pH 7.5) and then sedimented at 10,000 × *g* for 15 min at 4°C and resuspended in buffer A, which contained 50 mM HEPES-KOH (pH 7.5), 1 mM disodium EDTA, 2 mM dithiothreitol (DTT), 0.5% Triton X-100, 10% glycerol, and protease inhibitor cocktail tablets (one tablet per 10 ml; Roche Molecular Biochemicals, Indianapolis, Ind). Cells were broken with a French press (American Instrument Co., division of Travenol Laboratories, Inc., Silver Spring, Md.). The supernatant solution from centrifugation at 4°C for 20 min at 10,000 × *g* was subjected to centrifugation at 4°C for 1.5 h at 40,000 × *g*. Proteins were precipitated from the soluble supernatant solution by the addition of $(NH_4)_2SO_4$ to 30, 50, 70, and 90% saturation. All precipitates were dissolved in a small volume of buffer A and dialyzed overnight at 4°C against buffer B (50 mM HEPES-KOH [pH 7.5], 0.1 mM disodium EDTA, 2 mM DTT, 0.1% Triton X-100, 10% glycerol, protease inhibitor cocktail tablets), yielding respective protein fractions F30, F50, and F70 and F90. These fractions were stored at −80°C with 30% glycerol and were tested by gel shift assay (see below). Active fractions F50 and F70 were also combined and applied to a Hi-Trap heparin column (Amersham Biosciences Inc., Piscataway, N.J.), and protein fractions were eluted with a step gradient of 0.125, 0.25, 0.5, and 1.0 M KCl. The protein fractions collected were desalted by repeated concentration with Microcon YM-10 centrifugal filter devices (Millipore Corp., Bedford, Mass.) and redilution with 10 mM phosphate buffer (pH 8.0) and were then tested by gel shift analysis.

DNA affinity chromatography. PCR-amplified X12 (Table 2) oligomerized by self-ligation with T4 DNA ligase was coupled to CNBr-activated Sepharose (28), and the resulting sequence-specific DNA affinity resin was used to purify DNA-binding proteins as described previously (25). One milliliter of the DNA affinity resin thus generated was equilibrated in an Econo-Column (Bio-Rad, Hercules, Calif.) with 20 ml of buffer Z (25 mM K^+-HEPES [pH 7.8], 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol) containing 0.1 M KCl. Proteins partially purified by heparin column chromatography and extensively desalted and then diluted with 4 volumes of buffer Z containing 0.1 M KCl were allowed to bind for 1 h at 4°C with sonicated herring sperm DNA (20- to 50-fold excess by mass relative to the amount of X12 bound to the column) and then overnight with the DNA affinity resin. The protein-DNA mixture, 7 to 10 ml for one column, was allowed to pass through the Econo-Columns under gravity at ca. 15 ml/h. The resin in each column was then washed four or five times with 2 ml of buffer Z containing 0.1 M KCl. Protein bound to the column was eluted by two successive washings with 1.2 ml of buffer Z containing 1.0 M KCl. The eluted proteins were concentrated by precipitation with trichloracetic acid, the acid was removed with acetone, and the proteins were redissolved in buffer Z containing 0.05 M KCl and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with silver (23) or with Coomassie brilliant blue (41).

TABLE 2. Primer sequences used to generate DNA fragments by PCR

Gene or fragment	Primer sequence pairs[a]	Coordinates[b] on Anabaena sp. chromosome	Technique for which used
abp1 (*all1940*)	5′-CCCAT**ATG**GCAACAGTCGTTCTATGG-3′ 5′-CCGGATCC*TCA*TGCTTTTTTCGATAACAAC-3′	2325796–2325776 2324257–2324279	Overexpression
abp2 (*all1939*)	5′-CCCAT**ATG**AGGAAAGCAGCAACAG-3′ 5′-CCGGATCC*TTA*ATTCTGCGCTTGTTGTGG-3′	2324243–2324223 2322703–2322723	Overexpression
abp3 (*alr3608*)	5′-CCCAT**ATG**AAACATCAGGGGTTTAAC-3′ 5′-CCGGATCC*TTA*TTGGGGAACTTGAACTAG-3′	4358563–4358583 4360221–4360201	Overexpression
abp4 (*alr4240*)	5′-CCCAT**ATG**ACTCAACTCAATAGCGATC-3′ 5′-CCGGATCCTCAACTGTCAACTGTCAAC-3′	5083871–5083893 5085503–5085485	Overexpression
A1	5′-GCTCTAGAATTAGGTTTATCC-3′ 5′-GAAATGGATTGAATACAATCCTC-3′	3453779–3453796 3453856–3453834	Gel mobility shift assays
B2	5′-CCGAATTCTACAGAGCTTTGTTTCTCAG-3′ 5′-GATATGAATTCGACAACCTAATTTTTAAC-3′	3454104–3454123 3454193–3454176	Gel mobility shift assays
X1	5′-ATCGATTTTTTAACATAAATTGCC-3′ 5′-TACTTGTTTACTTGCACAATAATTTTC-3′	3452256–3452279 3452535–3452509	Gel mobility shift assays
X12	5′-CTATGACTATTTAAAGAG-3′ 5′-ACTTGTTTACTTGCACAATAATTTTC-3′	3452385–3452402 3452534–3452509	Gel mobility shift assays
X2	5′-CAAAGGTTAGGTTAATTGCC-3′ 5′-CGATTTTTAAAATCAGTATCC-3′	3452601–3452620 3452745–3452725	Gel mobility shift assays
Y	5′-GTCTTGATATAGGACTCATC-3′ 5′-TGTGTCATATTTGACATATC-3′	3098481–3098500 3098630–3098611	Gel mobility shift assays
abp1′	5′-CCCTGCAGCGCCAATTGCTCTCCAAC-3′ 5′-CCCTGCAGGCCAATTGTGCGATTTTC-3′	2325741–2325724 2324282–2324299	Insertional mutagenesis
abp2′	5′-CCCTGCAGCTAAAATTCAAAATGGC-3′ 5′-CCCTGCAGGTGACTTGAGCAGCTAATTG-3′	2324187–2324171 2322752–2322771	Insertional mutagenesis
abp3′	5′-CCCTGCAGGTTTTATCCTTACCAACTGG-3′ 5′-CCCTGCAGCCAGCATTGACTAATGCTTGG-3′	4358610–4358630 4360172–4360152	Insertional mutagenesis
abp4′	5′-CCCTGCAGGGGTTAGCTTGTGTGGGTATTG-3′ 5′-CCCTGCAGATTTTACGCACCTGTTTTAAAC-3′	5084067–5084088 5085440–5085426	Insertional mutagenesis
hepA (alr2835)	5′-ATGATTTTGGCTGCTGATGC-3′ 5′-CGAATTAGTCTGTCCTGATTCG-3′	3454463–3454482 3456058–3456037	Probe for Northern blot analyses
hepC (alr2834)	5′-ATGACAAGCGTAATAGTTCC-3′ 5′-GTAGGCTTGCGTTCCTGTAGG-3′	3452912–3452931 3453670–3453650	Probe for Northern blot analyses
rnpB	5′-GGCGTTGGCGGTTGCAGACC-3′ 5′-GGTGGTAAGCCGGGTTC-3′	4950801–4950782 4950371–4950387	Probe for Northern blot analyses

[a] For *abp1* through *abp4*, boldface ATGs serve as initiation codons and corresponding termination codons are italicized.
[b] Coordinates shown correspond to the region of sequence identity (http://www.kazusa.or.jp/cyano/Anabaena).

Protein sequencing. The single band of protein consistently observed upon SDS-PAGE was eluted. Sequence analysis of the proteins in that band was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase high-performance liquid chromatography–nano-electrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer with use of information about the *Anabaena* sp. strain PCC 7120 genome sequence (http://www.kazusa.or.jp/cyano).

Overexpression and purification of proteins Abp1, Abp2, Abp3, and Abp4. Genes *abp1*, *abp2*, *abp3*, and *abp4* were amplified by PCR (for primers, see Table 2). All PCR products were verified by sequencing. Corresponding products of PCR were cloned without a His tag (43, 49) between the *Nde*I (CATATG) and *Bam*HI sites (GGATCC) of plasmid pET-21b (Novagen, Inc., Madison, Wis.). The resulting plasmids were denoted, respectively, pRL2489, pRL2490, pRL2491, and pRL2492. To overproduce Abp1, Abp2, Abp3, and Abp4, *E. coli* strain BL21(DE3) transformed with each of these plasmids was grown in 250 ml of Luria-Bertani medium supplemented with 100 μg of ampicillin ml^{-1} at 37°C to an optical density at 600 nm of 0.5 to 0.6, 0.5 mM isopropyl-β-D-thiogalactopyranoside was added, and incubation was continued for 4 h at 37°C for Abp1, Abp2, and Apb3 and overnight at 22°C for Abp4. Following Frías et al. (17), these four overexpressed proteins were partially purified as follows. *E. coli* suspended in 50 mM Na phosphate–100 mM NaCl (pH 7.0) was broken with a French pressure cell. The supernatant solution from centrifugation at 4°C for 20 min at 10,000 rpm and then at 4°C for 20 min at 32,500 rpm was fractionated with ammonium sulfate. The protein fraction precipitating between 30 and 50% ammonium sulfate was used for mobility shift assays.

Gel mobility shift assays for DNA-protein complexes. Mobility shift assays (18, 19) were performed by using the DIG gel shift kit (Roche) according to the manufacturer's procedures. DNA fragments A1, B2, Y, X1, X12, and X2 used in these experiments were prepared by PCR with the primers shown in Table 2; double-stranded control oligonucleotide v6,

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5′-GTACGGAGTATCCAGCTCCGTAGCATGCAAATCCTCTGG-3′
    ||||||||||||||||||||||||||||||||||||||||
3′-CCTCATAGGTCGAGGCATCGTACGTTTAGGAGACCAGCT-5′
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is a component of the DIG gel shift kit. The DNA fragments were 3′-end-labeled by using DIG-11-ddUTP and terminal transferase. Labeled DNA probes (0.4 to 0.8 ng) were incubated with *Anabaena* sp. protein extracts in binding buffer (25 mM HEPES [pH 7.9], 5 mM $MgCl_2$, 25 mM NaCl, 0.5 mM DTT, 5% glycerol, 0.25 μg of bovine serum albumin μl^{-1}) containing 0.5 μg of poly(dI-dC) · poly(dI-dC) in a final volume of 20 μl. For gel shift competition, protein was allowed to bind with 100-fold excess by weight (100- to 194-fold molar excess), unless otherwise specified, of unlabeled competitor DNA for 15 min at room temperature before addition of the probe. Incubation with the labeled probe lasted for 20 min. The mixture was then loaded on an 8% polyacrylamide gel (30:1 acrylamide-bisacrylamide in Tris-glycine buffer containing 50 mM Tris, 380 mM glycine, and 2 mM EDTA [pH 8.5]) (45) which had been prerun for 1 h at 4°C in Tris-glycine buffer at 25 mA. Electrophoresis was performed at 4°C in Tris-glycine buffer for 2 to 2.5 h at 25 mA. The gel was then electroblotted onto Hybond-N+ nylon membranes (Amersham) at 200 mA in Tris-glycine buffer at 4°C for 1 h, the membrane was baked at 80°C for 2 h, and chemiluminescence was detected according to the instructions of Roche.

Insertional mutagenesis. Copies of the four *abp* genes, truncated at both ends (the truncation denoted with a prime sign), were prepared by PCR with the primers shown in Table 2 for *abp1′*, *abp2′*, *abp3′*, and *abp4′* and were treated with T4 DNA polymerase to blunt their ends. The resulting PCR copies were cloned between the *Sma*I sites of the polylinker of pRL498 (12) and then transferred by conjugation into wild-type cells of *Anabaena* sp. strain PCC 7120. Insertional mutants were selected on AA plus nitrate agar in the presence of 25 or 50 μg of neomycin ml^{-1}.

Luciferase assays. Luciferase activity of cyanobacterial cell suspensions was measured with a model TD-20/20 luminometer (Turner Designs, Sunnyvale, Calif.) (13) and was normalized to the concentration of chlorophyll *a* in the sample, which was measured in methanolic extracts (35).

RNA isolation and Northern blot analysis. Total RNA was isolated from wild-type and mutant cells of *Anabaena* sp. strain PCC 7120 by using TRIzol reagent (Life Technologies Division, Invitrogen Corp., Carlsbad, Calif.) according to the manufacturer's instructions. RNA samples were denatured and run on a 1.3% denaturing formaldehyde gel (30 μg/lane) in MOPS buffer (40 mM morpholinepropanesulfonic acid [pH 7.0], 10 mM Na acetate, 1 mM EDTA) and blotted onto Hybond-N+ nylon membranes (Amersham) according to standard procedures (41). Concentrations of RNA were measured spectrophotometrically at 260 nm before loading and were validated by the intensity of the ethidium bromide fluorescence from the rRNA bands observed under UV light before and after running the gels. RNA was fixed to the nylon membrane by baking at 80°C for 2 h. The blots were hybridized with α-^{32}P-labeled DNA probes prepared by random primer labeling and purified on Micro Bio-Spin P-30 Tris chromatography columns (Bio-Rad). The hybridization solution contained 7% SDS, 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2% blocking reagent (Roche), 50 mM Na phosphate buffer (pH 7.0), and 0.1% sodium *N*-lauroylsarcosinate (Sigma Chemical Co., St. Louis, Mo.). Hybridization was performed overnight at 42°C. Blots were washed twice for 5 min with 2× SSC–0.1% SDS at room temperature, twice for 10 min with 1× SSC–0.1% SDS at 57°C, and once for 10 min with 0.2× SSC–0.1% SDS at 57°C. Bands were quantified with a Molecular Dynamics (Amersham) PhosphorImager and Image Quant software. The radioactivity was plotted as counts per minute per square millimeter after background correction.

Probes for Northern blot analysis. DNA probes for *hepA*, *hepC*, and *rnpB* were prepared by PCR with the corresponding primers shown in Table 2.

Thin-layer chromatography of lipid extracts. Aliquots of methanol-chloroform (1:2, vol/vol) extracts of wild-type and mutant filaments, containing equal amounts of chlorophyll, were chromatographed on thin-layer chromatography plates precoated with silica gel (EM Reagents, E. Merck, Darmstadt, Germany) with a solvent system of chloroform-methanol-acetic acid-water (170:30:20:7.4, vol/vol) (38).

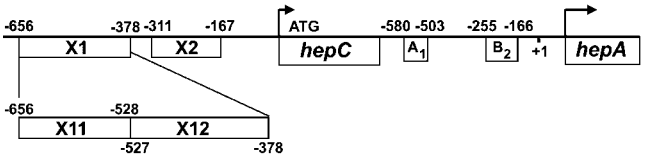

FIG. 1. Schematic representation of the region from bp 3452256 (on the left) to bp 3456061 (on the right) of the chromosome of *Anabaena* sp. strain PCC 7120 showing genes *hepC* and *hepA* and the origins of the DNA fragments used in this study. Numbers represent base pairs relative to the first ATG codon of *hepC* (for fragments X1, X2, X11, and X12) and relative to the transcriptional start site of *hepA* (for A1 and B2). DNA fragment X12 was used for protein purification.

RESULTS AND DISCUSSION

Proteins that bind specifically to a sequence upstream from *hepC*. DNA fragment X12 (Fig. 1) from *Anabaena* sp. strain PCC 7120 bound to proteins partially purified from that strain (Fig. 2, lanes 1 and 2). Lesser binding to fragments X11 and A1 will not be discussed further. Competition experiments with unlabeled DNA fragments A1, X2, and X12 (Fig. 1) showed that 100-fold excesses by weight (100- to 194-fold molar excesses) of fragments A1 and X2 had little or no effect on the formation of a complex between X12 and protein extract (Fig. 2, lanes 2, 7, and 8), but a 100-fold excess of X12 or X1 greatly reduced complex formation (Fig. 2, lanes 2, 3, and 6). Addition of a 100-fold excess of fragment B2 (Fig. 1) also reduced complex formation, albeit to a lesser extent (Fig. 2, lanes 2 and 5), perhaps due to the partial similarity of sequences X12 and B2 (Fig. 3).

DNA-binding proteins purified from extracts of *Anabaena*

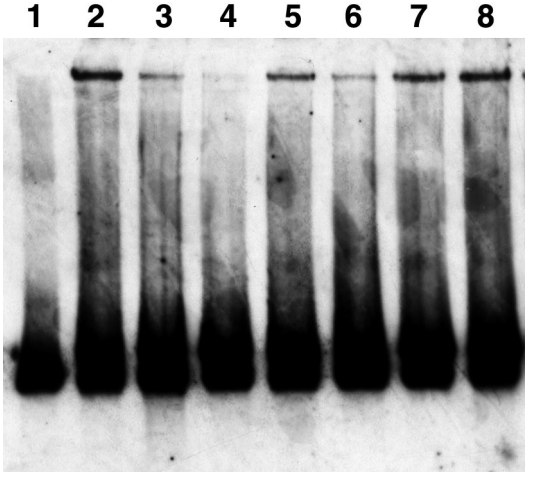

FIG. 2. Mobility shift assay. Protein extract partially purified from *Anabaena* sp. strain PCC 7120 by precipitation between 50%- and 70%-saturated $(NH_4)_2SO_4$ binds specifically to sequence X12. Labeled X12 was used as the probe with the following additions. Lanes: 1, no added protein; 2, protein extract; 3, protein extract plus 100-fold nanogram excess of unlabeled X12; 4, protein extract plus 400-fold nanogram excess of unlabeled X12; 5, protein extract plus 100-fold nanogram excess of unlabeled B2; 6, protein extract plus 100-fold nanogram excess of unlabeled X1; 7, protein extract plus 100-fold nanogram excess of unlabeled A1; 8, protein extract plus 100-fold nanogram excess of unlabeled X2. The shifted bands are near the top of the photographic image, which lies beneath the loading wells of the gel.

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          1                                                  50
      X12 ........CT ATGACTATTT AAAGAGTAAA TAAAGAATTA GCTTGAGTAT
        Y GTCTTGATAT AGGACTCATC TTTGA.TTTC TGAAAAACCA AATTGGCTAG
       B2 .......... .......... .......... .......... ......
Consensus .........t a.gact..t. ...ga.t... t.aa.aa..a ..ttg..ta.

          51
      X12 ATAGTCATTT CTACAGAGTC TTATATCCTA GAGATTGAAA TCTAATTCCA
        Y A.AGCTTGCT ATACAGAGCT TTGTTTCTCA GTATACGTAG CAAAATTCAA
       B2 .......... .TACAGAGCT TTGTTTCTCA GTATACGTAG CAAAATTCAA
Consensus a.ag.....t .TACAGAGct TTgTtTCtcA GtatacGtAg caaAATTCaA

          101
      X12 ATGGTTTCAA AGTAGAAATG AACTGATGGT CTGAAAATTA TTGTGCAAGT
        Y GTACGATTCC TATATGATTT TTCAGTATGC CTGATA.... .TGTCAAATA
       B2 GTACGATTCC TATAGA.... ...TGAATTA CTACACTTTA GTTAAAAATT
Consensus gTacgaTtcc taTAgaa.t. ..ctGaatg. CTgaaa.tta .Tgt.aAAtt

          151
      X12 AAACAAGTA
        Y TGACACA..
       B2 AGGTTG...
Consensus agaca....
```

FIG. 3. Comparison of the sequences of fragments X12, B2, and Y, aligned with Multalin (http://www.toulouse.inra.fr). Proteins Abp1, Abp2, Abp3, and Abp4 bind specifically to each of these fragments.

sp. vegetative cells by a combination of ammonium sulfate precipitation (only fractions F50 and F70 bound X12), heparin column chromatography, and DNA affinity chromatography on a column of oligomerized X12 DNA coupled to CNBr-activated Sepharose 4B consistently showed a single principal band of protein of 56 to 58 kDa in SDS-PAGE gels (Fig. 4). Sequence analysis of the proteins isolated from this band showed the presence of polypeptides corresponding to four different basic proteins, denoted Abp1, Abp2, Abp3, and Abp4 (named for *Anabaena* sp. DNA-binding protein) (Table 3), with molecular mass as expected (Table 4). Two of these proteins (Abp1 and Abp2) were encoded by neighboring genes in the *Anabaena* sp. chromosome. The genes that encoded the third (Abp3) and fourth (Abp4) proteins were located elsewhere (http://www.kazusa.or.jp/cyano/Anabaena).

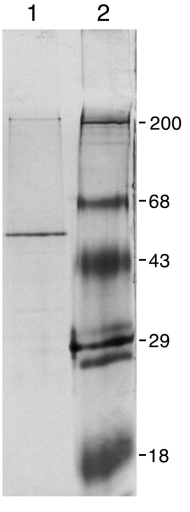

FIG. 4. SDS-PAGE analysis, on a 10% polyacrylamide gel, of proteins eluted from specific-DNA affinity chromatography. The gel was stained with silver. Lane 1, protein purified by using the oligomerized X12 fragment of DNA coupled to CNBr-activated Sepharose; lane 2, molecular masses of protein standards in kilodaltons.

TABLE 3. Characteristics of polypeptides obtained from the Harvard University Microchemistry Facility

Sequence identified	TIC[a]	Ions[b]	Protein
VENSPIGMMVER	5.4e7	14/22	Abp1
ATFTPENR	8.2e7	9/14	Abp1
FLDPVIR	1.1e8	10/12	Abp1
LAQTYFGR	1.4e8	11/14	Abp1
VPTSIQPYLDR	3.5e8	16/20	Abp1
FDYPGM*FM*AGGQTR[c]	2.3e7	16/26	Abp2
ITEYATINAIAR	2.8e8	18/22	Abp2
VSPANTGGVFFVNQPQL TQSSILVGHLGGK	2.7e8	31/116	Abp2
HYTELQFAPLPEVK	1.3e8	21/52	Abp2
ELPLIGGTALVR	2.3e8	19/22	Abp2
NPQFTKPTLPK	2.5e8	15/20	Abp2
QHSPDDLNQILEQR	3.9e8	23/52	Abp2
AEIAAFVYQALVNAGR[d]	3.6e7	21/30	Abp3
	1.7e8	33/60	Abp3
EGVFVTGTGNPK	1.1e9	17/22	Abp3
DGIVATVNAQPDLGTSE NPGGNLIR	6.9e7	19/48	Abp3
IDSFPFSQFGDIK	9.9e6	16/24	Abp4

[a] TIC (total ion current) of the MS/MS spectrum; it does not distinguish a major from a minor component.
[b] Number of fragment ions observed out of the number possible.
[c] *, + 16.000 modification of the preceding amino acid.
[d] This peptide was represented by two scan numbers.

We wished to determine whether one or more of these proteins regulates (i) transcription of *hepC* and, perhaps indirectly, *hepA* and (ii) differentiation of heterocysts. Cells of *Anabaena* sp. strain PCC 7120 to which truncated copies of all four *abp* genes were transferred were tested for growth in the presence and absence of fixed nitrogen. Mutants in which *abp2* or *abp3* was inactivated grew well in the presence of nitrate but yellowed rapidly and fragmented when deprived of fixed nitrogen. Both mutants grew again when transferred to AA/8 plus nitrate medium. *abp1* and *abp4* mutants grew both with and without nitrate. Southern blot hybridization experiments (data not shown) showed complete segregation of all four kinds of mutants.

Single recombination of pRL1069a with *Anabaena* sp., producing derivative SR1069a, placed *Vibrio fischeri luxAB* under the control of the *hepA* promoter while retaining the ability of the strain to fix N_2 aerobically. Northern blot hybridization experiments showed that expression of *hepC* and *hepA* is induced after 10 h of nitrogen deprivation of cells of wild-type *Anabaena* sp. (Fig. 5A and B) or of strain SR1069a (Fig. 6A) but not of SR1069a derivatives CPB8226 (mutated in *abp2*) and CPB8563 (mutated in *abp3*) (Fig. 5A and B and 6B and C). Increasing activity of luciferase was observed in cells of strain SR1069a but not in the *abp2* and *abp3* mutant derivatives of strain SR1069a (Fig. 6C). It is clear from growth experiments (data not shown) that genes *abp2* and *abp3* are unimportant during growth on nitrate but required for growth on N_2. Surprisingly, electron microscopy has shown that *abp2* and *abp3* mutants ABP2 and ABP3, despite their weak expression of *hepA* and *hepC*, form an envelope polysaccharide layer but neither a laminated envelope layer of glycolipids (Fig. 7) nor heterocyst envelope glycolipids, as shown by thin-layer chromatography of lipid extracts (Fig. 8). Evidently, even very weak expression of *hepA* and *hepC* suffices for the production of

TABLE 4. Characteristics of four *Anabaena* sp. proteins

Protein	Abp1	Abp2	Abp3	Abp4
Encoding gene (chromosomal coordinates)[a]	*all1940*, also known as *abp1* (2324258–2325844)[a]	*all1939*, also known as *abp2* (2322703–2324241)	*alr3608*, also known as *abp3* (4358563–4360221)	*alr4240*, also known as *abp4* (5083872–5085443)
No. of amino acids	512	512	552	523
Molecular mass (kDa)	57.754	56.782	58.272	58.748
Theoretical pI	7.74	9.61	9.41	8.93
Motif	Insulinase family, zinc-binding	None noted	S-layer	Leucine zipper
BLAST search	Presumptive protease of *Synechocystis* sp. strain PCC 6803 (D90914; BLAST score, 584; E = 10^{-166}; identities, 60%; positives, 77%)	Presumptive processing proteinase of *Synechocystis* PCC sp. strain 6803 (S75528; BLAST score, 496; E = 10^{-139}; identities, 54%; positives, 72%)	S-layer associated multidomain endoglucanase (AAD09354) from anaerobic thermophile *Thermoanaerobacterium polysaccharolyticum* (BLAST score, 105; E = 10^{-21}; identities, 34% positives, 57%)	Hemolysin HlyD-like ATP-binding cassette protein of *Synechocystis* sp. strain PCC 6803 (BLAST score, 340; E = 2×10^{-92}; identities, 40%; positives, 58%)

[a] Information can be found at http://www.kazusa.or.jp/cyano/Anabaena.

FIG. 5. Expression of *hepC* (A) and *hepA* (B) derived by quantitation of Northern blots of mutants CPB8226 and CPB8563 in Fig. 6 and of wild-type *Anabaena* sp. and luciferase activity (ATP photometer units per microgram of chlorophyll *a*) of the *hepA*::*luxAB* transcriptional fusions in strain SR1069a and in its derivatives, CPB8226 and CPB8563 (C).

heterocyst envelope polysaccharide. In the *abp1* and *abp4* mutants ABP1 and ABP4, which grow with or without nitrate in the medium, *hepA* and *hepC* are expressed but are induced only after 24 h of nitrogen deprivation, much later than they are in the wild-type strain (data not shown). Although not essential for heightened expression of *hepC* and *hepA*, Abp1 and Abp4 evidently play a minor role in the regulation of transcription of those two genes.

The four *abp* genes, cloned in vector pET-21b, were overexpressed in *E. coli*. All four partially purified Abp proteins bound specifically to and retarded fragment X12 in gel shift experiments (Fig. 9). They also bound to sequences B2 and Y

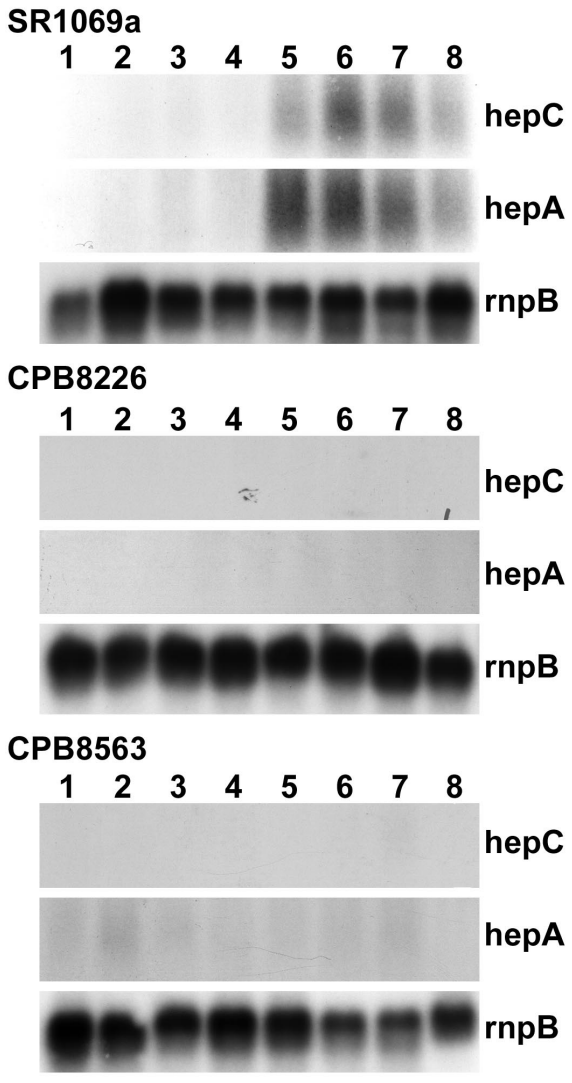

FIG. 6. Transcripts of *hepC* and *hepA* in derivatives SR1069a, CPB8226, and CPB8563 of *Anabaena* sp. strain PCC 7120. Northern hybridization with the *hepC* and *hepA* probes (see Materials and Methods) shows induction of expression of corresponding genes in 10 h in response to nitrogen deprivation of cells of derivative SR1069a of *Anabaena* sp. (the same result was obtained for cells of wild-type *Anabaena* sp. [see Fig. 6A and B]). No induction of expression was seen in the *abp2* (CPB8226) and *abp3* (CPB8563) derivatives of strain SR1069a. An *rnpB* (44) probe was used as a loading control. Lanes: 1, cells grown with nitrate, unwashed; 2, cells washed; 3 through 8, washed cells deprived of fixed nitrogen for 3, 6, 10, 24, 30, and 48 h, respectively.

(see below; data not shown), although not to the other DNA sequences tested. Sequences of X12 and B2 (Fig. 3) have a region of similarity that may account for the fact that the four proteins bound to both of these sequences. A similar sequence, denoted Y (Fig. 3), was found elsewhere in the genome, in the middle of a gene cluster that may be involved in iron transport. The region of similarity is downstream from open reading frame (ORF) *alr2590*, whose predicted product shows similarity to an Fe^{3+} dicitrate-binding periplasmic protein, and upstream from a presumptive transcriptional regulatory gene, *alr2591*, and a presumptive ferrichrome-iron receptor gene, *alr2592* (26).

Sequence similarities of the DNA-binding proteins. Comparison of the amino acid sequences of Abp1 through Abp4 with those of other proteins showed the following. Abp1 and Abp2 are most similar (respective BLAST scores, 584 and 496; Expect values [E] = 10^{-166} and 10^{-139}) (1) to a presumptive protease and a processing proteinase, respectively (GenBank accession no. D90914 and S75528), of *Synechocystis* sp. strain PCC 6803 (Table 4). Abp1 and Abp2 show extensive similarity also to probable proteases of *Pseudomonas aeruginosa*, *Rhizobium* spp., *Streptomyces coelicolor*, *Deinococcus radiodurans*, and *Bacillus subtilis*. Abp1 also possesses the signature {amino acids 79 through 102, GGVDEPDGKTGVA**H**FLE**H**-LAFKGT [ScanProsite web site (http://www.ebi.ac.uk/interpro/scan.html)]} of a zinc-binding region, where the two H's (boldface) are ligands to zinc. Abp2 is similar to a hypothetical zinc protease, Y4WB (P55680) of *Rhizobium* sp. strain NGR234 (BLAST score, 131; E = 2×10^{-29}) but appears not to contain a zinc-binding site (ScanProsite website). That each of two proteins that bind specifically to fragment X12 would show similarity to proteases was unexpected.

Abp2 shares similarity (E = 7×10^{-10}) with the mitochondrial processing peptidase-like protein, Mpp, of *Bradyrhizobium japonicum*, and it was recently shown that a *B. subtilis* homolog, MlpA, of mitochondrial processing peptidases may regulate the transcription and/or secretion of a group of proteins (4). The authors discuss the possibility that MlpA binds upstream from and represses one of the regulated sequences but consider this possibility unlikely because MlpA lacks known DNA-binding motifs. From our results, this possibility may warrant further consideration. Alternatively, the authors suggest, MlpA may modulate the activity of a transcriptional regulator. It is tempting to speculate that Abp1 and Abp2, which are encoded by neighboring genes, bind DNA, and resemble proteases, act together.

More than 300 different species of eubacteria and archeabacteria possess a regularly arranged structure, named the S (surface)-layer, which covers the cell envelope. S-layers are paracrystalline monolayered assemblages of proteins, some of which are glycosylated or phosphorylated (42). Several S-layer proteins and some other cell wall proteins contain one or more copies of a domain of about 50 to 60 residues, known as an S-layer homology domain, which may anchor the protein to the peptidoglycan (32, 34). Alternatively, O-polysaccharide side chains of the lipopolysaccharide component of the cell walls of gram-negative bacteria may serve as receptors for the attachment of the S-layer (5). Abp3, which contains three S-layer homology domains (amino acids 360 through 402, 420 through 462, and 482 through 526) (ScanProsite website), showed similarity (BLAST score, 105; E = 10^{-21}) to an S-layer-associated multidomain endoglucanase of an anaerobic bacterium, *Thermoanaerobacterium polysaccharolyticum* (Table 4). Unlike Abp1, Abp2, and Abp4, whose indicated similarities extend throughout the proteins, only about one-third of Abp3 showed similarity to less than 20% of that endoglucanase. Interestingly, the S-layer-like protein, SlpM, of *Thermus thermophilus* HB8 is one of three proteins that control the expression of *slpA*, the gene that encodes the protein that comprises the crystalline surface layer (15). Insertional inactivation of *slpM*

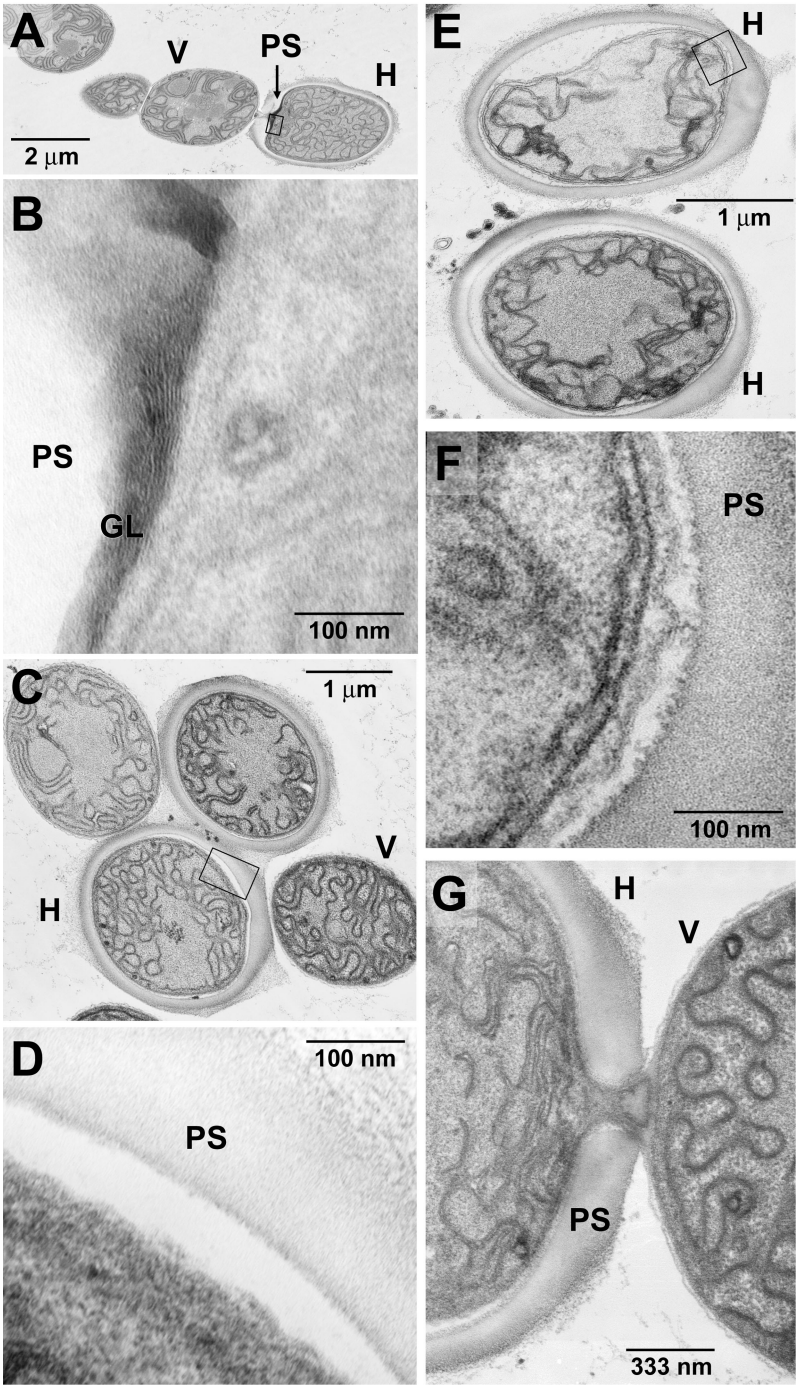

FIG. 7. *abp2* and *abp3* mutants form a heterocyst envelope polysaccharide layer but no glycolipid layer. (B [a magnification of the boxed area in panel A]) In a heterocyst of wild-type *Anabaena* sp., the laminated layer of glycolipids (47) (the laminations vertically arrayed in panel B) is enveloped by a layer of polysaccharide. In contrast, the only envelope layer seen in a heterocyst of an *abp3* mutant (D [a magnification of the boxed area in panel C]) or an *abp2* mutant (F [a magnification of the boxed area in panel E] and G) is the polysaccharide layer. H, heterocyst; V, vegetative cell; GL, glycolipid layer; PS, polysaccharide layer.

demonstrated its in vivo function in the control of *slpA* transcription: SlpM acts as an activator. Notably, SlpA itself acts as a specific repressor of its own translation by binding directly to the 5′-untranslated region of its own transcript.

Abp4 is most similar (BLAST score, 340; $E = 2 \times 10^{-92}$) to a hemolysin secretion-like protein from *Synechocystis* sp. strain PCC 6803 (Table 4) and to related ATP-binding-cassette transporters ($E = 8 \times 10^{-22}$ and 6×10^{-20}). Abp4 is encoded by the central ORF of three closely spaced ORFs (http://www.kazusa.or.jp/cyano/Anabaena) that may constitute an operon. The predicted products of the other two ORFs are similar, respectively, to a *Synechocystis* sp. protein that resembles a

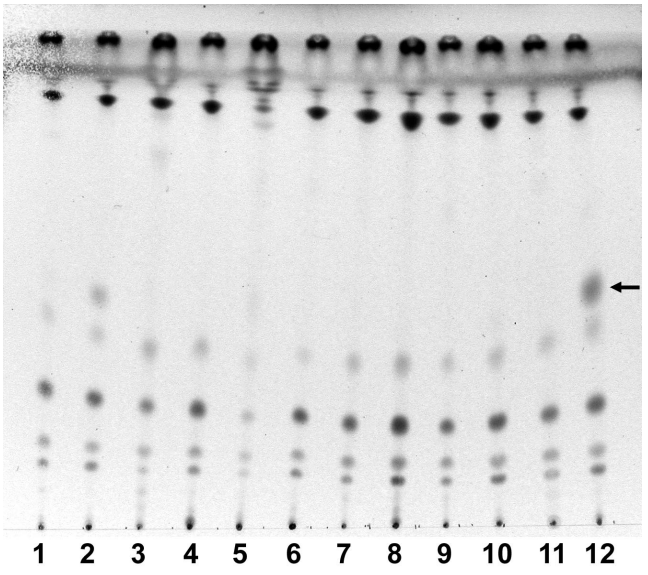

FIG. 8. Thin-layer chromatography of lipid extracts shows that heterocyst envelope glycolipids are absent from *abp2* and *abp3* mutants. A lipid extract of wild-type *Anabaena* sp. (lanes 1 and 2) and its derivative strains ABP2 (lanes 3 and 4), ABP3 (lanes 5 and 6), SR1069a (lanes 11 and 12), CPB8226 (lanes 9 and 10), and CPB8563 (lanes 7 and 8) were chromatographed. The cyanobacteria had been grown in the presence (odd-numbered lanes) or in the absence (even-numbered lanes) of nitrate in the medium. The arrow marks heterocyst-specific glycolipids.

hemolysin secretion protein (BLAST score, 1,021; $E < 10^{-180}$) and to the protein predicted by ORF *slr0655* of *Synechocystis* sp. (BLAST score, 196; $E = 10^{-49}$).

Protein Abp4 contains three leucine (bold L) zipper motifs: **L**EFVRSR**L**PQEATF**L**LRSRAA**L**, corresponding to amino acids 167 through 188, **L**PQEATF**L**LRSRAA**L**VAENEL**L**, corresponding to amino acids 174 through 195, and **L**TQTTVK**L**QDTQSS**L**AIQQRI**L**, corresponding to amino acids 241 through 262. Leucine zipper motifs are present in many proteins with diverse functions and are characteristic of some eukaryotic DNA-binding, gene regulatory proteins (8, 31). The leucine side chains facilitate dimerization of proteins (39) and appear to be involved in protein-protein interactions.

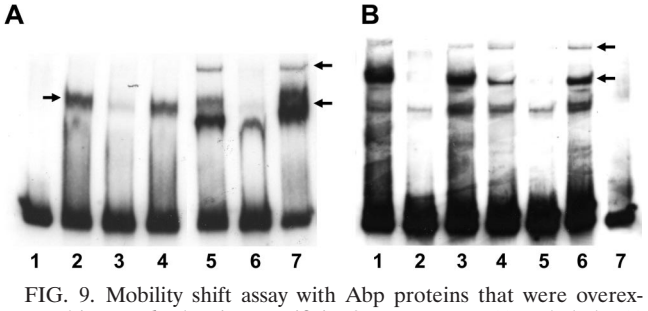

FIG. 9. Mobility shift assay with Abp proteins that were overexpressed in *E. coli*, showing specificity for sequence X12. Labeled X12 was used as the probe, with the following additions: (A) Lanes: 1, no added protein; 2, Abp2 protein extract; 3, Abp2 protein extract plus 100-fold nanogram excess of unlabeled X12; 4, Abp2 protein extract plus 100-fold nanogram excess of unlabeled v6 (see Materials and Methods); 5, Abp3 protein extract; 6, Abp3 protein extract plus 100-fold nanogram excess of unlabeled X12; 7, Abp3 protein extract plus 100-fold nanogram excess of unlabeled v6. (B) Lanes: 1, Abp1 protein extract; 2, Abp1 protein extract plus 100-fold nanogram excess of unlabeled X12; 3, Abp1 protein extract plus 100-fold nanogram excess of unlabeled v6; 4, Abp4 protein; 5, Abp4 protein plus 100-fold nanogram excess of unlabeled X12; 6, Abp4 protein plus 100-fold nanogram excess of unlabeled v6; 7, no added protein. The arrows point to labeled DNA-protein complexes whose formation was reduced after addition of unlabeled DNA fragment X12.

In conclusion, we have identified four proteins that bind sequence specifically to DNA of *Anabaena* sp. strain PCC 7120, even though none has a known DNA-binding domain and all bear extensive similarity to proteins that have other known roles. Mutants lacking two of these proteins show developmental defects and greatly reduce expression of *hepC* and *hepA*, suggesting that these proteins indeed function as transcriptional regulators. Our data add new examples to other examples that have been reported (2, 11, 30, 40) of proteins that lack a known DNA-binding domain but nonetheless bind sequence specifically to DNA and regulate transcription.

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